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THE EFFECT OF ULTRASONICS ON THE PERMEABILITY OF A LIVING MEMBRANE TO NA^{22}

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FOREWORD

The investigation reported herein was conducted by the Biology Department of The Catholic University of America research project "Research on the Action of Ultrasound on Cell Membranes" supported by the USAF under Contract No. AF 33(616)-438, RDO No. R 695-63, with Major Horace O. Parrack, Project engineer.

This investigation was a prerequisite basic for research on living cell membranes. This effect was tested using a radioactive isotope to measure the effect by altering the permeability of the living cell membrane. The results of radioactivity and ultrasound appear to be the same in their effect on cell permeability but the mechanism may not be the same.

A systematic evaluation of the applications of ultrasound to medicine has only begun. It is certain that this radiation produces heat in the body tissues; however, whether purely mechanical or other non-thermal effects play a significant role has yet to be determined.

Acknowledgement is made to Prof. Pulvari, H. F. Mengoli, M. E. Gilson and C. Rice for their contributions in the successful pursuit of this project and also to the Atlas Powder Company, Wilmington, Delaware, for contributing the various surface active agents used in the experiments.

ABSTRACT

This report introduces some preliminary tests necessary to pursue research on the effects of ultrasound on living cell membranes. By means of an isotope it was possible to measure the permeability of a living cell membrane to a weak ultrasonic field. Techniques and instrumentation had to be developed. As a result of the experiments described, it has been shown that a weak ultrasonic field does increase the permeability of the living cell membrane of the amoebae in much the same way as do surface active agents, lipase and thioglycollic acid.

PUBLICATION REVIEW

This report has been reviewed and is approved.

FOR THE COMMANDER:

JACK BOLLERUD
Colonel, USAF (MC)
Chief, Aero Medical Laboratory
Directorate of Research

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF ILLUSTRATIONS

Tables — Page

SECTION I

INTRODUCTION

The accumulation of information regarding living cell membranes to the present time, seems to indicate that they are made up of long molecular chains arranged regularly in a network composed of micelles and intermicellar spaces. It is supposed that all cell membranes consist of these micelles. In animal cells, however, the micellar structure is probably composed of a lipoid-protein complex. The lipoid layer is thought to be several fatty molecules thick with an adsorbed protein layer at each water-lipoid interface. This fatty membrane of extreme delicacy completely surrounds the cell. In a system such as is found in a cell, the diffusion of metabolites inwards and outwards across its boundary will be a very essential part of its metabolic process. Therefore, the composition and structure of the membrane is of paramount importance. Diffusion is materially hindered by the presence of this membrane. From what is known of its structure, it is possible to conceive that the lipoid molecules are capable of arranging themselves in a most restrictive manner (1). The long protein chains are thought to be held together partly by chemical bonds and partly by van der Waals forces (2) (3).

It is conceivable that anything which will dissolve the fatty components of the membrane should remove their restrictive character and bring about an increase in permeability. In the same manner, anything that will weaken the van der Waals forces holding together the long protein chains will bring about an increase in permeability. By varying the amount of dissolved lipoid as measured by permeability, it should be possible to determine the amount of lipoid present in the membrane. Similarly, by applying varying degrees of force it should be possible to investigate and eventually to determine the strength of these forces holding together the protein chains and perhaps ultimately, the length and nature of these chains by analogy.

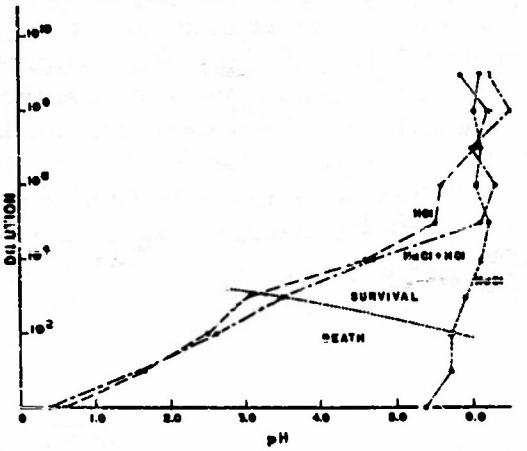
It was first proposed to test the lipoid nature of living cell membranes by means of surface active agents whose hydrophilic and lipophilic characteristics had already been determined. Following this, it was proposed to investigate by means of varying forces produced by ultrasound, the strength of the forces holding together the proteins, i.e., to cause a change in permeability similar in nature to the permeability change caused by dissolving away a certain amount of lipoid. It is believed that both processes cause an increase in permeability and some correlation was looked for between the two changes in order to obtain some information about the nature and function of such a living cell membrane.

A measurement of these phenomena has been accomplished by means of rates of permeability traced through a radioactive isotope known to be relatively impermeable to the living cell membrane. The isotope chosen for these tests was Na^{22} and the animal cell was Pelomyxa carolinensis.

SECTION II

MATERIALS AND METHODS

Since Na^{22} was available only in the form of the salt in a weak solution of HCl, it was necessary to determine the viability of Pelomyxa carolinensis in various dilutions of NaCl, HCl and the combination of NaCl and HCl.



It can be seen from the graph (Figure 1) that HCl was more toxic than NaCl, but that when the two chemicals were combined, they were no more toxic than HCl alone. In all tests using either or both chemical agents, amoebae survived in dilutions of 10^{-3} for at least twelve hours. Determination of the pH of each solution (Table I) revealed that amoebae survived in a lower pH in HCl alone and in the combination of HCl and NaCl than in NaCl alone.

Figure 1. Toxicity of various dilutions of NaCl, HCl, and NaCl:HCl on Amoebae.

TABLE I

pH of Various Dilutions ofChemicals Used

Dilutions	NaCl	HCl	NaCl:HCl
10-1	5.39	0.61	0.41
10-2	5.70	1.60	1.61
10-3	5.71	2.50	2.60
10-4	5.90	3.01	3.50
10-5	6.10	4.59	4.70
10-6	6.20	5.51	6.10
10-7	6.05	5.58	6.30
10-8	6.10	6.10	6.01
10-9	6.00	6.20	6.50
10-10	6.10	5.84	6.21

The isotope selected was a new product of Na^{22}, therefore it was necessary to determine its general characteristic in respect to this investigation. This isotope was found to be more active dry than when wet (Table II, Figures 2 and 3) and glass as well as water reduced, as expected, the effect registered on the Geiger Counter (Table III and Figure 4). Therefore, it was decided to dry each sample and subsequent counts were made under those conditions. After treating amoebae with the isotope it was necessary to find out how many washes were needed to remove this agent adhering to the surface of the amoebae. Three washes in distilled water removed the isotope as proved by the results recorded in Table IV. However it was decided to wash at least six times and to use the sixth wash of each sample as background count. We regarded the background count as the number of beta decays registered on the counter when no isotope was present.

TABLE II

The Difference in cts/min/ml of a Wet and Dry Sample
(Tube distance 3 in. for a 1 ml. sample, 10^{-3} dilution)

(a) Wet Sample : 3767 cts/min/ml
(b) Dry Sample : 10,057 cts/min/ml

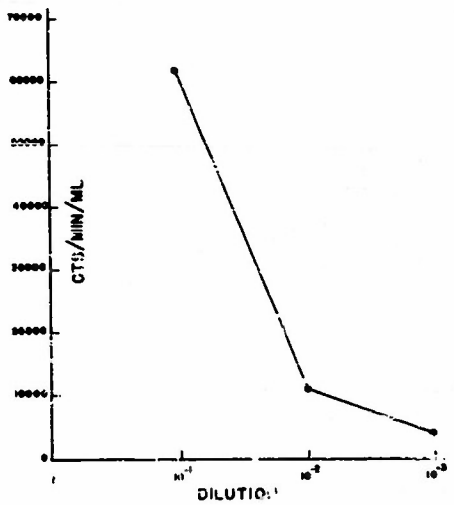


Figure 2. Radioactivity of samples of Na^{22} (wet) at various dilutions.

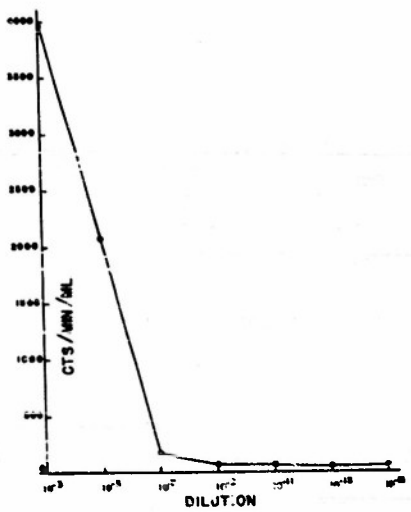


Figure 3. Radioactivity of samples of Na^{22} (dried) at various dilutions.

TABLE III

Radioactivity of Amoebae Samples in Dry State after Immersion (45 min, Na^{22} 10^{-1} dilution, with and without cover slip and after water was added to dried sample).

(a) Without coverslip : 346 cts/min
(b) With one no. 1 cover slip: 117 cts/min
(c) With two no. 1 cover slips: 45 cts/min
(d) With three no. 1 cover slips: 17 cts/min
(e) With four no. 1 cover slips: 10 cts/min
(f) Without cover slip but with water added approximately equal to water evaporated. Ref. (a): 77 cts/min

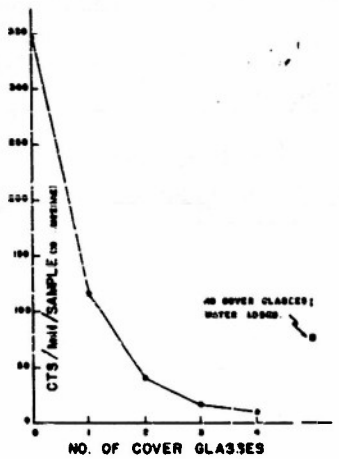


Figure 4. Radioactivity of Amoebae sample (Dried) after placing cover slips between sample and counter.

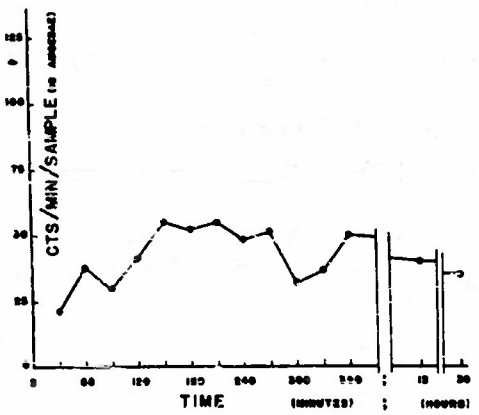


Figure 5. Uptake of Na^{22} by Amoebae immersed in isotope solution for various periods and dried.

TABLE IV

Number of Washes Necessary to Remove All Na^{22} Adhering to Outer Surface of Amoebae (Immersion Time 2 hr. 10^{-3} dil.).

(a) 10 amoebae	130 cts/min/sample
(b) 1st wash	1948 cts/min/sample
(c) 2nd wash	56 cts/min/sample
(d) 3rd wash	counts negligible
(e) 4th wash	counts negligible
(f) 5th wash	counts negligible
(g) 6th wash	counts negligible
(h) 7th wash	counts negligible

TABLE V

Uptake of Na^{22} by Amoebae at Various Time intervals after Immersion (Na^{22}, dil. 10^{-3}).

(a) 30 min	21 cts/min/sample
(b) 60 min	38 cts/min/sample
(c) 90 min	30 cts/min/sample
(d) 120 min	41 cts/min/sample
(e) 150 min	55 cts/min/sample
(f) 180 min	53 cts/min/sample
(g) 210 min	55 cts/min/sample
(h) 240 min	47 cts/min/sample

(i) 8th wash counts negligible
(j) Original 10^{-3} dilution
38,246 cts/min/sample

(i) 270 min 52 cts/min/sample
(j) 300 min 32 cts/min/sample
(k) 330 min 36 cts/min/sample
(l) 360 min 50 cts/min/sample
(m) 13 hrs 40 cts/min/sample
(n) 30 hrs 35 cts/min/sample

After these determinations had been made the amount of isotope taken up by amoebae without any treatment was tested. Ten amoebae at a time were placed in a 10^{-3} dilution of the isotope for 30 minute intervals up to six hours; after washing six times in distilled water, samples were dried and counted. It was found that amoebae took up Na^{22} throughout this time interval but the uptake remained fairly constant (Table V and Figure 5). In all the tests, the uptake of the isotope by untreated amoebae was very slight compared with those samples of treated amoebae.

SECTION III

PROCEDURE AND RESULTS

If membrane permeability is increased as a result of the weakening of the intermolecular bond strength, then, anything which affects this will be important during the measurement of permeability. Horton (1) treated bacteria with ultrasound to test the ultrasonic death rate. He found that as the surface tension decreases, the ultrasonic death rate decreases. Both ionic and non-ionic surface active agents (contributed by the Atlas Powder Company, Wilmington, Delaware) were employed in this investigation to determine their effect on the membrane's permeability. The non-ionic agents increased the permeability of the amoebae to Na^{22} after one hour (Table VI). The rate of uptake of Na^{22} was greater in the Tween series which reduced surface tension more than did the Span compounds.

TABLE VI.

Uptake of Na^{22} by Amoebae after Treatment With Surface Active Agents.

Time	Tween 20 (10^{-3}) cts/min/10 amoebae	Tween 40 (10^{-2}) cts/min/10 amoebae	Tween 60 (10^{-3}) cts/min/10 amoebae
5 min	21	20	33
10 min	36	44	71
15 min	82	67	130
30 min	70	80	214
45 min	97	96	300
60 min	129	100	305

Time	Tween 80 (10^{-2}) cts/min/10 amoebae	Span 20 (10^{-4}) cts/min/10 amoebae	Span 80 (10^{-4}) cts/min/10 Amoebae
5 min	33	30	21
10 min	63	28	35
15 min	68	74	34
30 min	138	38	61
45 min	179	78	113
60 min	257	95	128

An interesting correlation resulted in the study of individual permeability tests. It was noted that the non-ionic surface active agents altered permeability in a manner which suggested that alteration depended more on the relationship of the agents to the lipoid in the membrane than in the HLB grouping. As may be seen in Figures 6 and 7, those compounds which contained oleic acid (Tween 80 and Span 80) increased permeability more than those containing lauric acid (Tween 20 and Span 20). Tween 60 (Figure 8) brought about the greatest uptake of Na^{22}. This surface active agent contains stearic acid. Since the fatty component of the membrane seems to dissolve more in this agent containing stearic acid than in any of the other acid groups, this may account for the greater permeability of the membrane whose structure may include a substance soluble in this acid. This appears to conform to the theory of Overton (4) in which he suggested that the permeability of the cell membrane might depend upon the solubility of the substance of which the membrane is composed.

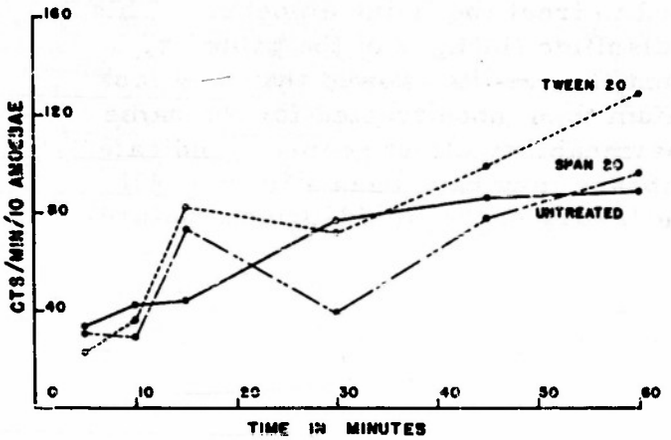


Figure 6. Radioactivity of Amoebae untreated and treated with Tween 20 and Span 20.

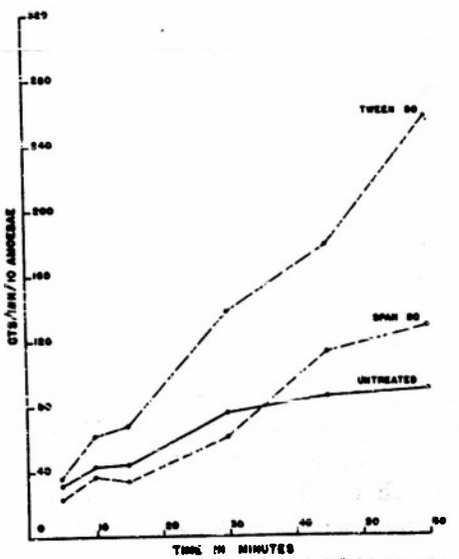


Figure 7. Radioactivity of Amoebae untreated and treated with Tween 80 and Span 80

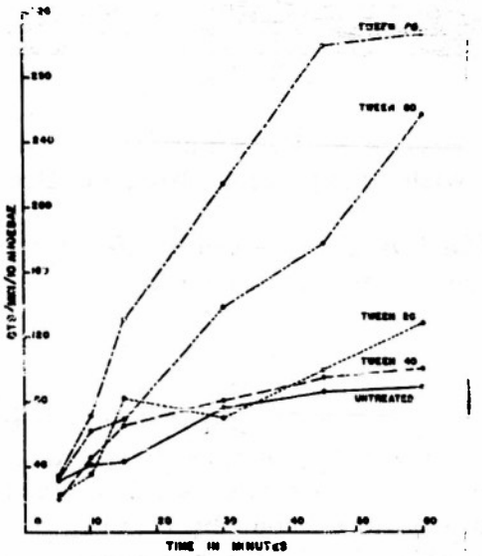


Figure 8. Radioactivity of Amoebae untreated and treated with Tween 20, 40, 60 and 80

In another test using lipase, which is known to be specific for lipoids, the amoebae took up more Na^{22} than after similar treatment with surface active agents (Figure 9). This appeared to indicate that the theory of the lipoid entering into the structure of the membrane was correct. Since the membrane is also supposed to be partly protein in nature, thioglycollic acid was used to treat the living amoebae. This agent is specific for disrupting the disulfide linkages of the proteins. After treating amoebae with this agent, the results showed that they took up one and one-half times more sodium than those treated for the same time with lipase (Figure 9). This permeability effect seems to indicate that the protein complex of the membrane may have been altered. All of these tests at least strengthen the theory of the lipoid-protein nature

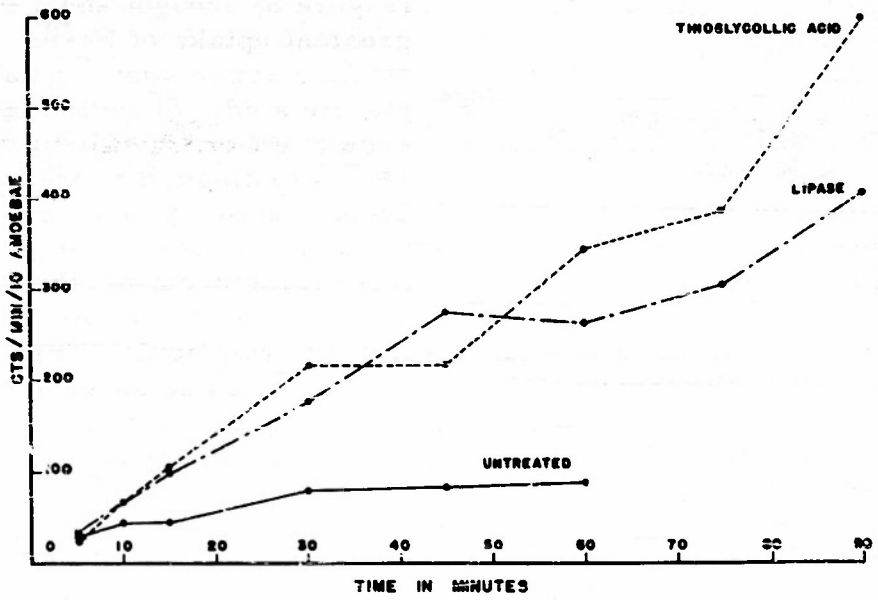


Figure 9. Radioactivity of Amoebae untreated and treated with lipase and thioglycollic acid.

of the living cell membrane of the amoebae and show that by various chemical agents the membrane may be altered so as to increase its permeability to Na^{22}.

These initial experiments appeared to indicate the lipoid-protein nature of the living cell membrane of Pelomyxa carolinensis. The molecules of this membrane are then apparently bound by chemical bonds and van der Waals forces. Both of these, presumably, could be perturbed by ultrasound. However, the van der Waals force probably could be most easily perturbed. Experimental work, however, has indicated that denaturation of proteins (5) can be effected by ultrasound.

Most of the work done by investigators with ultrasonics in biology has been conducted to bring about lethal or fragility reactions (6, 7, 8, 9, 10). The present tests were devised using an ultrasonic field in which amoebae would survive during and after treatment but, at the same time, would be affected by the treatment. Therefore, in the following experiments, amoebae were subjected to a weak ultrasonic field at five megacycles.

A x-cut, gold-plated, quartz-crystal was driven at its lowest resonant frequency by a high frequency generator. The H.F. transmitter was connected to the quartz crystal placed in a separate brass holder; the outer surface of the crystal was immersed in a bath of distilled water, and a concave reflecting surface placed opposite the face of the crystal to focus the ultrasonic waves at the water surface. Amoebae were placed in this focussed area in a container fashioned from a piece of lucite tubing one inch in diameter and one centimeter long. One end of the tubing was grooved to hold a "number one" cover glass which was cemented to it to form the bottom of the container. The container, with the amoebae resting on the bottom glass in a 10-3 dilution of the isotope, was placed in the weak ultrasonic field for different periods of time: 1, 5, 10, 15, 30, 45, and 60 minutes. This field has been estimated to be around 1 watt/cm^2 For each period, 10 amoebae were treated with the weak field which did not produce turbulence or disturb the amoebae on the bottom of the container. After treatment, the amoebae were washed in six washes of distilled water. The sixth wash was dried and checked for activity and used as the background count for that sample as previously defined. Amoebae were then measured for uptake of Na^{22}. The test was repeated four times, and in all tests when the Geiger counter was used, at least ten counts of each sample were made. The average of ten counts for each time interval and the average deviation, were computed to the nearest tenth. All counts were made with a Tracerlab Geiger-Mueller Tube TGC-2 and Tracerlab SC-19 Utility Scaler with an operating voltage of 1350 volts.

After amoebae had been treated with a weak ultrasonic field, the cells showed an increased permeability to the isotope as compared with cells untreated for the same period of treatment (Table VII).

TABLE VII

Uptake of Na^{22} by Amoebae Immersed in 10^{-3} Dilution after Treatment in a Weak Ultrasonic Field

Time	Av. cts/min. 10 amoebae
1 min	68
5 min	88
10 min	139
15 min	105
30 min	136
45 min	172
60 min	110
10^{-3} dilution of isotope	35,181 cts/min/0.1 ml

During the first 10 minutes permeability increased rapidly; then there appeared to be a leveling off or an adjustment period, after which the uptake of Na^{22} by the amoebae reached its peak after 45 minutes (Figure 10). Although a decrease in permeability was noted after the peak was reached at 45 minutes, the uptake of Na^{22} did not drop as low as that observed for the untreated amoebae.

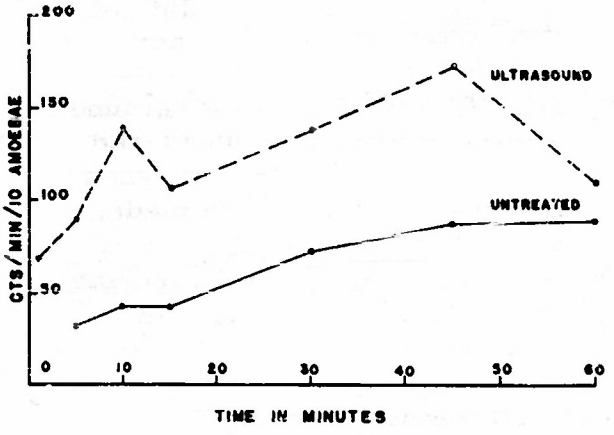


Figure 10. Radioactivity of Amoebae untreated and treated with ultrasound.

Further investigation will be required to determine whether perturbation of the van der Waals forces or denaturation of proteins, or both of these phenomena, occurred in the present series of tests. Detailed quantitative methods are needed to establish more precisely the nature of the molecular structure of the membrane. A measurement of the pressure field with a newly developed pressure gauge will be possible in future tests using ultrasound. Photographs of the assembled pressure gauge (consisting of a cathode follower and sensing element) are shown in Figures 11 and 12.

Figure 11. Experiment equipment ready for use.

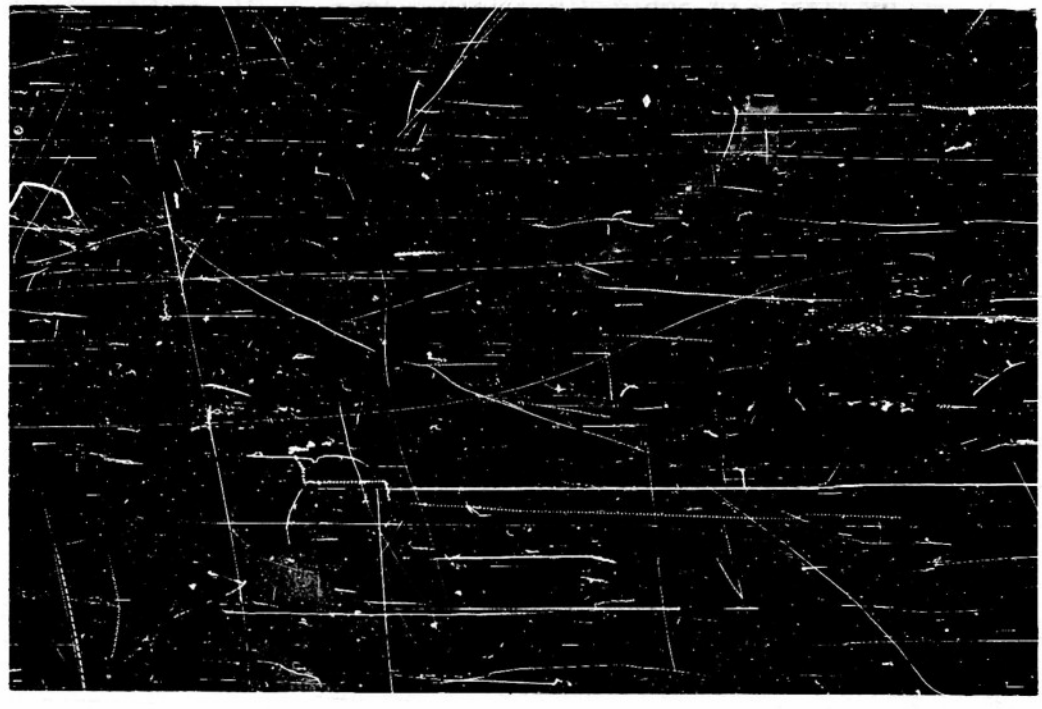


Figure 12. Close up of transducer and pressure gauge.

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